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Muramic Acid Is Not Detectable in *Chlamydia psittaci* or *Chlamydia trachomatis* by Gas Chromatography-Mass Spectrometry

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By using the powerful separation technique of capillary gas chromatography combined with the selectivity of mass spectrometric detection, muramic acid was not detectable in purified elementary bodies of *Chlamydia psittaci* Cal 10 (≤0.006%) or *C. trachomatis* serovar E (≤0.02%). This confirms previous reports which suggested the absence of a typical peptidoglycan in *Chlamydia* spp.

Chlamydia spp. were originally thought to resemble viruses because of their requisite intracellular growth in eucaryotic cells. However, identification of both DNA and RNA in these organisms (1) and demonstration of their sensitivity to penicillin, chloramphenicol, and tetracycline indicated they were of bacterial origin (9). More recently, 16S rRNA sequencing of *C. psittaci* has confirmed the eubacterial origin of this organism (20).

Penicillin generally displays bactericidal or bacteriostatic activity by inhibiting cross-linking of peptidoglycan, often resulting in lysis of the cell wall. Matsumoto and Manire (13) showed convincingly by transmission electron microscopy that the presence of penicillin in cultures of *C. psitacci*-infected L cells results in interruption of reticulate body binary fission. This was confirmed by Barbour et al. (2) in an elegant study in which they also identified three penicillin-binding proteins in the cytoplasmic membrane (or cytoplasm) of both the elementary body and the reticulate body in the lymphogranuloma venereum biovar of *C. trachomatis*.

Recently, in one of our laboratories, in examining the nonoxidative antimicrobial effects of human polymorphonuclear leukocyte granule proteins in vitro, it was found that fractions containing lysozyme caused reductions in infectivity of *C. trachomatis* (>70%). Lysozyme is a muramidase that generally functions by lysing the linkages between *N*-acetyl muramic acid and *N*-acetylglucosamine in the glycan backbone of peptidoglycan. However, lysozyme could have cellular targets other than peptidoglycan, and in some instances, effects might not be related to muramidase activity (14, 16).

The peptidoglycan of all eubacteria studied to date has been shown to contain muramic acid. In contrast, archaebacteria have a pseudomurein that contains *N*-acetyltalosaminuronic acid but not muramic acid, while mycoplasma totally lack a cell wall and muramic acid. Muramic acid is also not synthesized by eucaryotes, including fungi and mammals (5, 7, 17).

Early reports noted detection of muramic acid, albeit in very small amounts, in both *C. psitacci* and *C. trachomatis* harvested from embryonated eggs (10, 15). These reports used colorimetric methods of analysis, which often cannot readily distinguish between low levels of chemically related compounds present in a complex mixture such as chlamydiae partially isolated from the contents of embryonated eggs. For example, glucosamine and other amino sugars present in large amounts in glycoprotein can cause interferences in the colorimetric analysis of muramic acid. This is particularly the case when muramic acid is present only at trace levels.

The development of tissue culture cell lines for growth of chlamydiae and improved methods for obtaining more purified preparations of elementary and reticulate bodies and their isolated cell walls stimulated a new search for muramic acid in *Chlamydia* spp. (2, 6, 11, 12). These newer studies also used improved analytical techniques, which included chromatographic separation of muramic acid from other sugars and amino acids, but conflicting results persisted. In one study, *C. trachomatis* was found not to contain muramic acid, although it was readily detected in gram-negative bacteria including *Escherichia coli* (2). *C. psittaci* was not analyzed in that study. In another chromatographic study also using chlamydiae isolated after growth in a tissue culture cell line, the presence of muramic acid in *C. trachomatis* was suggested (6). The latter study was also the first to report the absence of muramic acid in *C. psittaci*. Table 1 summarizes the results of several studies on muramic acid levels in *Chlamydia* spp.

Over the past 10 years, we have been concerned with developing a gas chromatographic-mass spectrometric procedure for detecting low concentrations of muramic acid obtained from bacterial debris present in mammalian tissue (5, 7). Capillary gas chromatography is a powerful separation technique, particularly when combined with the extreme selectivity of mass spectrometric detection. In view of the continuing controversy as to whether muramic acid is present in different chlamydial species, we investigated this important question.

The Cal 10 meningopneumonitis strain of *C. psitacci* was grown in L-929 suspension cells by the method of Tamura and Higashi (18). Elementary bodies were harvested at 48 h postinfection. The *C. trachomatis* biovar E/UW-5/Cx was grown in McCoy cell monolayers in roller bottles. Infected cells were harvested at 96 h. Intracellular organisms were released by sonication of infected cells and were pooled with the cell-free supernatants. Elementary bodies were purified by differential centrifugation and by centrifugation through 30% sucrose cushions and discontinuous gradients of 38, 44, and 59% Renografin-76 (E. R. Squibb & Sons, Princeton, N.J.), as previously described (16). To remove all traces of

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TABLE 1. Summary of previous searches for muramic acid in chlamydiae

Organism	Growth and isolation[a]	Presence of muramic acid	Reference
C. psittaci Cal 10	DCE	+[b]	10
C. psittaci			
Pigeon isolate	DCE	+	15
Feline pneumonitis	DCE	+	15
C. trachomatis			
Mouse pneumonitis	DCE	+[b]	15
Trachoma	DCE	+[b]	15
C. psittaci Cal 10	DCE	−(<0.2%)[c]	11, 12
C. psittaci Cal 10	DGCTC	−(<0.04%)	6
C. trachomatis LGV[d] (fast strain)	DGCTC	+[e]	6
C. trachomatis LGV	DGCTC	−(<0.02%)[c]	2

[a] DCE, Differential centrifugation from egg; DGCTC, density gradient centrifugation of tissue culture.
[b] Determined by colorimetry.
[c] Determined by amino acid analysis.
[d] LGV, Lymphogranuloma venereum biovar.
[e] Determined by thin-layer chromatography of radioactive muramic acid.

Renografin (methylglucamine), used as a standard in muramic acid analyses, the purified elementary bodies were dialyzed repeatedly. The maximum total particle counts in different preparations analyzed were 8.6×10^{11}/ml for *C. psittaci* and 3.6×10^{11}/ml for *C. trachomatis*.

Replicate samples of *C. psittaci* (4 mg each on one occasion and 10 mg on another), *C. trachomatis* (3 mg each on one occasion and 1 mg on another), 26 strains of legionellae, including *Legionella pneumophila* and 7 other species of legionellae (2 to 10 mg each), and *Candida albicans* (5 to 10 mg) were analyzed in duplicate. The legionellae and *C. albicans* were grown in charcoal yeast extract and Sabouraud broth, respectively. Each culture was heat killed, washed in distilled water, and lyophilized. The dry weights of chlamydiae present in suspensions were calculated from a protein assay, assuming whole cells were 50% protein; other microbial samples were available as lyophilized powders. The details of the gas chromatographic-mass spectrometric analysis have been described previously (4). In brief, each sample was hydrolyzed in 2 N sulfuric acid. Each mixture was neutralized with *N*,*N*-dioctylmethylamine (Fluka Chemical Corp., Haupage, N.Y.). The aqueous phase was passed through a C-18 column to remove hydrophobic contaminants (J. T. Baker Chemical Co., Phillipsburg, N.J.) and reduced with sodium borohydride. Acetic acid-methanol (1:200, vol/vol) was added, and the sample was dried repeatedly to remove borate, which otherwise inhibits the acetylation reaction. The samples were acetylated with acetic anhydride (Alltech Associates, Inc., Deerfield, Ill.) at 100°C overnight and then extracted with acid and alkali to remove polar contaminants. Analyses were carried out by using a 5890 gas chromatograph containing an SP-2330 fused silica capillary column (SGE, Austin, Tex.) and interfaced to a 5970 mass spectrometer (Hewlett-Packard Co., Palo Alto, Calif.).

At the retention time for muramic acid in chromatograms of *C. trachomatis*, no peak above background levels was seen. The maximum amount of muramic acid present was calculated to correspond to no more than 0.02% of the dry weight of whole chlamydial cells. In chromatograms of *C. psittaci*, muramic acid was present at no more than 0.006% on a dry weight basis. There was a threefold-lower detection limit for *C. psittaci* because three times the dry weight of material was available. A yeast, *C. albicans*, served as a negative control. Background peaks were tiny or absent and of the same order of magnitude in chlamydial and fungal chromatograms. *L. pneumophila* and other legionellae served as positive controls. In agreement with previous work, muramic acid was readily detected in the range of 0.1 to 0.3% in all legionellae analyzed (3, 8, 19). Members of the family *Legionellaceae* contain comparable amounts of muramic acid to other gram-negative bacteria. Figure 1 shows chromatograms of alditol acetates of whole cell hydrolysates of *C. psittaci* compared with *L. pneumophila* and *C. albicans*. It should be emphasized that the *L. pneumophila* sample contained 10 times the amount of internal standard (methylglucamine) as *C. psitacci* to allow for accurate quantitation. After taking into account this correction factor, it can be seen that the muramic acid peak area is 242-fold larger in the *L. pneumophila* chromatogram as compared with the *C. psittaci* chromatogram. In conclusion, the present study confirms that muramic acid is not detectable in either *Chlamydia* species (levels were less than 0.02% for *C. trachomatis* and less than 0.006% for *C. psittaci*). We suggest that the potential presence of a small amount of muramic acid would be unlikely to provide a functional peptidoglycan. The explanation for the activities of penicillin and lysozyme, whose target is usually peptidoglycan, against chlamydiae remains to be determined.

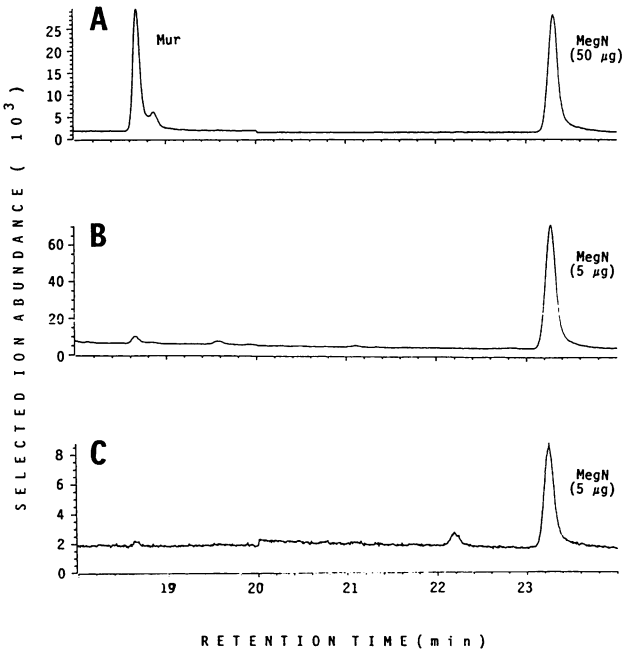

FIG. 1. Enlarged portions of selected ion-monitoring chromatograms of alditol acetates from hydrolysates of whole cells of *L. pneumophila* (A), *C. psittaci* (B), and *C. albicans* (C). Separations were performed by using an SP-2330 fused silica capillary column. Analyses were performed in the electron impact ionization mode. Mur, Muramic acid; MegN, methylglucamine (internal standard). When comparing chromatograms by using the amount of methylglucamine as a reference, it should be recognized that the samples in panels B and C contained 10 times less methylglucamine than that in panel A.

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